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METHOD FOR ENUMERATION OF THIOBACILLUS FERROOXIDANS

Phase I - Literature Review

for

Energy, Mines and Resources, Canada

by

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SUMMARY

Phase I of a research program directed at the development of a simple and direct method for the enumeration of *Thiobacillus ferrooxidans* in slurries has been completed. A critical assessment has been undertaken of existing methodologies including most probable number (MPN) methods, direct plate count, direct microscopic count and indirect methods. The major limitation(s) of each method has been identified. The development of a more accurate enumeration method will depend on the identification of a technique to quantitatively separate the *T. ferrooxidans* from the particulate material with which it strongly associates.

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1. INTRODUCTION

This report (to end of Phase I) is submitted in accordance with the terms of DSS Contract Serial No. OSQ. 83-00198 and as outlined in the work statement of our Proposal # P-4488/G.

2. OBJECTIVE

The objective of the overall program is to develop a simple and direct protocol for the enumeration of Thiobacillus ferrooxidans in slurries and suspensions of sulphide-containing particles. The objective for Phase I has been to undertake a critical assessment of the state-of-the-art of methods for the enumeration of T. ferrooxidans. Based on our observations, an experimental strategy will be developed (Phase II) in conjunction with CANMET personnel for implementation as Phase III of the total program.

3. LITERATURE SEARCH

A computer-assisted literature search was done. Key articles and secondary references were then consulted. The references consulted are listed in the bibliography (Appendix I) and the supplement (Appendix II). The latter section includes those articles which were reviewed but are not referred to in the text of this report.

4. GENERAL OVERVIEW

T. ferrooxidans is an autotrophic bacterium which has both a beneficial and detrimental role in the mining environment. This unusual situation occurs because of the organism's relatively unique ability to thrive in a low pH environment with a high metallic content and grow on completely inorganic nutrients. It catalyzes the dissolution of sulphide components of ores by oxidizing both ferrous iron and reduced sulphur compounds to produce acidic ferric sulphate. This reaction is the basis of biological leaching processes for the recovery of metals from sulphide-containing ores. However, when this acidic ferric sulphate solution is generated in sulphide-containing tailings by the action of endogenous organisms, it then contributes to the serious pollution problem of acid mine drainage.

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Additional microbial species, such as Thiobacillus thiooxidans and related thiobacilli, also play a role in the biological leaching process and the generation of acid mine drainage. Some organisms appear to have an indirect ecological role whereas the thiobacilli, which are capable of oxidizing reduced sulphur compounds to produce sulphuric acid, play a more direct role. However, T. ferrooxidans remains of particular interest because of the iron-oxidizing ability by which it can initiate and maintain the generation of acid ferric solutions from the oxidative dissolution of pyrite-containing minerals.

The economic and environmental importance of T. ferrooxidans has stimulated considerable research since its isolation from coal mine drainage waters in 1947 (Colmer and Hinkle, 1947). A series of excellent reviews, which describe the significance of T. ferrooxidans, has been published (Brierley, 1978; Kelly et al., 1979; Torma, 1977; Lundgren et al., 1972). However, much fundamental knowledge about this organism has still to be gained in order to improve the control of both biological leaching processes and acid mine drainage.

There remains significant gaps in the understanding of the precise mechanism by which this microbe can catalyze the dissolution of sulphide minerals (particularly iron pyrite) with the resultant generation of sulphuric acid. Direct attack by the bacteria on mineral sulphides is thought to occur, but this aspect is still a matter for debate. There is a need, therefore, to elucidate further the nature of the interaction of the Thiobacillus with the mineral ore surfaces. Such information will assist the bioengineer to determine the extent to which the cell's physiology might be manipulated in order to optimize the efficiency of biological leaching processes.

The management of tailings for the prevention of acid generation (and the subsequent leaching of toxic metals) would benefit if a method were available to monitor more accurately the changes occurring in the T.ferrooxidans population within the tailings. By the time that acid

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leachates are observed, the microbial activity within the tailings is well established. It is then more difficult to alleviate the problem. Early-warning techniques are required to permit management personnel to use control measures on tailings, in advance of the observed chemical changes. This should not only be more effective but also economically more attractive to the mining companies.

Related to both the leaching processes and acid mine drainage is the need to develop a further understanding of the microbial ecology of T. ferrooxidans. Evidence has been obtained which suggests a synergistic relationship between T. ferrooxidans and acidophilic heterotrophs (Kingma and Silver, 1981; Arkesteyn and DeBont, 1980). However, there is little knowledge concerning microorganisms which are antagonistic to the growth of T. ferrooxidans. Furthermore, little has been described about the biological mechanisms involved in the successive changes in microbiological populations which eventually lead to a predominance of acidophilic organisms.

Progress in acquiring this key information has been greatly impeded by the inadequate methodology for enumeration of T. ferrooxidans cells in slurries (Brierley, 1978). Nevertheless, many research groups have used procedures such as the most probable number (MPN) technique in order to estimate the magnitude of populations of T.ferrooxidans. In some instances, reservations about the methodology have been noted by the authors (Schubert and Miller, 1982) and a recent study has indicated that the MPN procedure was unreliable when used to enumerate T.ferrooxidans in pyrite-containing tailings (Silver and Sanmugasunderam, 1983). In later sections of this report, we identify why current methods yield inaccurate results and require very long incubation periods. Consequently, until an improved method is available to enumerate T.ferrooxidans in samples containing particles, studies to assess the effects of genetic, physiological and ecological forces on the behaviour of T.ferrooxidans will be awkward to perform and difficult to interpret.

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In this phase we have concentrated on the critical evaluation of the acquired information as it relates to the enumeration of T.ferrooxidans in slurries containing relatively high percentages of particulate material. An effective technique must address two key factors namely:

a) Factors influencing the growth and metabolism of T. ferrooxidans.

b) The nature of the interaction of T. ferrooxidans cells with particles.

These topics will influence, either directly or indirectly, the accuracy of all types of enumeration methods and they will therefore be reviewed in separate sections.

They will be followed by a critical review of the current methods used for the direct enumeration of T. ferrooxidans including most probable number (MPN) counts, plate counts, and direct microscopic counts. Finally, the possible application of indirect methods in direct enumeration technology will be discussed.

In our evaluation we have considered factors such as the techniques for the release/dispersion of T. ferrooxidans adsorbed on to the surfaces of particles and the effect of the presence of other species of acidophilic, iron-oxidizing microorganisms on the accuracy of an enumeration method. Relevant information on these aspects will be noted in the general text.

Secondary factors such as the mineralogy of ores and the effect of ore-processing parameters have also been included but the level of development of the enumeration methodology does not warrant an in-depth review of those secondary factors at this point in time. They will be important when a potential methodology has to be validated with natural samples of differing compositions.

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5. CELL GROWTH AND METABOLISM IN T. FERROOXIDANS

T. ferrooxidans is an autotrophic, gram negative, rod-shaped bacterium. The genus *Thiobacillus* includes organisms distinguished by their ability to derive their energy from the oxidation of reduced sulphur compounds (Buchanan and Gibbons, 1974). Because enumeration methods for viable cells usually require cell growth and/or an active metabolism, some consideration must be given to factors which influence these activities in *T. ferrooxidans*.

5.1. Carbon Sources

The carbon source for *T. ferrooxidans* is CO_2, which is fixed via the Calvin pathway (Gale and Beck, 1967). In cultures where CO_2 is in excess, growth is limited by the availability of ferrous iron (as an energy source), while in cultures where the CO_2 level is low, CO_2 becomes the limiting factor for growth. Carbon dioxide, however, is not a requirement for oxidation of ferrous iron, as it has been demonstrated that the oxidation can continue in the absence of CO_2 at a linear rate for at least 140 hours (Kelly and Jones, 1978).

T. ferrooxidans is considered to be a strict autotroph and does not grow on organic sources of carbon. Organic compounds have, in fact, been shown to be inhibitory to the growth of the organism (Tuttle and Dugan, 1976; Murakami *et al.*, 1977). Adaptation of *T. ferrooxidans* to growth on organic compounds has been reported (Gale and Beck, 1967; Shafia *et al.*, 1972; Tabita and Lundgren, 1971), but these results may be artifacts caused by contamination of the cultures with acidophilic heterotrophs (Guay and Silver, 1975; Silver, 1978).

5.2. Energy Sources

T. ferrooxidans can derive energy by the oxidation of both ferrous ion or reduced sulphur compounds. In this respect, it is unique from other thiobacilli which can only oxidize sulphur compounds (Buchanan and Gibbons, 1974). *T. ferrooxidans* is capable of growing on elemental sulphur as well as a variety of reduced forms of sulphur such

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as thiosulphate, tetrathionate, and thiothionate. The organism can be cultured for long periods in sulphur medium and then will adapt to grow in ferrous iron medium. Similarly, the organism is able to adapt to grow on sulphur medium after having been grown on ferrous iron medium (Tuovinen and Kelly, 1974b). Prolonged cultivation, however, on sulphur compounds is facilitated by adding a trace concentration of iron (Eccleston and Kelly, 1978).

While growing on ferrous iron, T. ferrooxidans will tolerate high concentrations of other cations. Thus, it has been suggested that specific binding sites for ferrous ions exist on the cell envelope. (Takakuwa et al., 1977).

In iron-containing medium, the levels of both ferrous and ferric ion appear to be significant. Studies by Kelly and Jones (1978) have indicated that in batch cultures of T. ferrooxidans, the production of ferric ion by bacterial oxidation of ferrous ion can become the main limiting factor to cell growth. This phenomenon of end product inhibition appears to involve a competitive mechanism and it does not affect CO_2 fixation.

It was also found (Kelly and Jones, 1978) that high potassium concentrations can partially offset this inhibition. Other research groups (Yunker et al., 1981; Belyi and Gurevich, 1976), however, overcame this effect by constructing an electrolytic culture vessel in which the ferric ion is converted electrochemically to the ferrous form. This type of reactor prevents the accumulation of the inhibitory end products and regenerates the supply of ferrous ions for further bacterial energy requirements. Using such a device, Belyi and Gurevich (1976) determined that for optimum growth in batch cultures, ferric ion concentration should be less than 13g/L; the concentration of ferrous ion should be 3 - 5g/L; and the ratio between ferric and ferrous ions should be maintained at the optimum. Kovrov and Denisov (1978) determined with such an electrolytic vessel that the rate of ferrous ion oxidation

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increased directly with increasing ferrous iron concentration but inversely with total iron (ferrous + ferric) concentration.

5.3. Nitrogen Sources

While nitrogen requirements for T. ferrooxidans are usually supplied by ammonium ions, a strain of this species has also been described which is capable of fixing dinitrogen gas (Kelly and Jones, 1978). Some strains also appear to be capable of using urea (Brierley and Brierley, 1968).

Tuovinen et al. (1979), studied the influence of a variety of nitrogen compounds on ferrous oxidation by two strains of T. ferrooxidans. They found with both strains, that ammonium was a limiting nutrient for ferrous oxidation. One strain was capable of using nitrate, but not nitrite, as a nitrogen source. Seven different amino acids (tested at 0.5mM) either inhibited or had no effect on iron oxidation. Complex nitrogen sources such as beef extract, casein, malt extract, peptone, tryptone, and yeast extract had an inhibiting effect at 0.1 and 0.5%(w/v) but a stimulating effect at 0.005 and 0.01%(w/v) respectively. In the absence of a nitrogen source, ferrous oxidation was linear for at least 160 hours.

5.4. Oxygen

Oxygen is essential to the growth of T. ferrooxidans (Buchanan and Gibbons, 1974). At an optimal pH (approx. 2.5), the limiting oxygen concentration for this bacterium's growth was examined over a range of 25°C to 34°C and found to be approximately 5% of the oxygen concentration at saturation (Myerson, 1981).

5.5. Other Chemical Requirements

Other major nutrient requirements such as phosphorus and sulphate are obtained in the form of inorganic salts (Lazaroff, 1963; Dugan and Lundgren, 1965). Schnaitman et al. (1969) have demonstrated that sulphate requirements can be partially satisfied by phosphate or arsenate ions.

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T. ferrooxidans requires a number of trace elements such as potassium and calcium ions but there is also evidence that trace levels of organics may be beneficial. For example, Wakao et al. (1983) observed that the rate of pyrite oxidation was increased many times by the addition of very low quantities (approx. 0.001% w/v)of organic materials. This stimulatory effect was observed even for substances which are not metabolized by T. ferrooxidans. It was therefore suggested that the effect of these materials is to facilitate a physico-chemical interaction of the bacteria and solid particles.

5.6. pH

The growth limits of T. ferrooxidans occur between pH 1-5 (Silverman and Ehrlich, 1964), while the cells are inactivated at pH 9 (Malouf and Prater, 1961). Good growth occurs between pH 1.5 to 3.5 with an optimum of approximately 2.5 (Guay et al., 1975; MacDonald and Clark, 1970). Depending on the nutrient content of the growth medium, the pH optimum may change. For example, growth on thiosulphate is optimal at pH 3.6 (Tuovinen and Kelly, 1974b).

5.7 Temperature

Although the optimum temperature for the growth of T. ferrooxidans is approximately 28^{o}C., the optima for biochemical processes oxidizing metallic sulphates and ferrous iron are higher, ranging up to 35^{o}C. (Silverman and Lundgren, 1959a, 1959b; Guay et al., 1975; MacDonald and Clark, 1970). Razzell (1962) reported that outside a temperature range of 5^{o}C to 37^{o}C., sulfide leaching by T. ferrooxidans is not practical.

5.8. Toxic Effects

As mentioned previously T. ferroxidans is a strict autotroph and is inhibited by the presence of organic materials except at low levels. Brierley (1978) noted that the tolerance of T. ferrooxidans to toxic metals "varies with the strain" although information on the tolerances of the numerous strains was limited. Nevertheless, it can be stated that, compared to many bacteria, T. ferrooxidans appears relatively resistant to toxic metals.

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The reaction of a given strain of T. ferrooxidans to metal toxicity will depend on its history and the organism can be adapted to display increased resistance towards metals. Silverman and Ehrlich (1964) reported that freshly isolated cultures of T. ferrooxidans were sensitive to high concentrations of metallic ions but adapted rapidly and became resistant to 5,000 ppm or more of copper, ferrous or ferric iron, aluminum, zinc, molybdenum, cobalt, uranium, and other metals. Other laboratories (Furyaeva et al., 1976; Olson et al., 1981; Sugio et al., 1981; Tuovinen and Kelly, 1974a) have also reported the adaptation of T. ferrooxidans to increased concentrations of heavy metals. Mercury and silver ions are particularly toxic to T. ferrooxidans (Imai et al., 1975) and as little as 5×10^4 mM Hg has been shown to inhibit this organism (Brierley, 1978). Molybdenum above concentrations of 0.03 mM. (5 mg/L) is inhibitory to the growth of T. ferrooxidans; yet, if this organism behaves in similar manner to T. thiooxidans in sulphur media, trace levels may be stimulatory (Takakuwa et al., 1977).

Anionic detergents, which may be present in samples of mining process effluents from flotation units, have also been shown to be toxic to T. ferrooxidans. Detergents such as sodium dodecyl sulphate and sodium dodecyl benzene sulphonate are inhibitory in the range 10 to 40 ppm (Kleinmann et al., 1981; Garcia et al., 1981).

The presence of particles in a sample may also have an inhibitory effect. Dispirito et al. (1981) observed inhibition of T. ferrooxidans growth at pH 1.5 upon the addition of particles of fluorapatite, glass beads, pyrite, and flowers of sulphur. Except for pyrite, whose inhibitory effect appeared to be caused by a soluble compound, the toxic effect required direct contact with the particles. The authors suggested that bacterial activity at a liquid-solid interface is different than in the bulk liquid phase.

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6. ASSOCIATION OF T. FERROOXIDANS WITH PARTICULATE MATERIAL

Association of bacterial cells with particulate matter is a fundamental problem with enumeration techniques. In methods where serial dilutions are required, sampling errors can result due to the heterogeneous nature of the cell-particle suspension. Thus, although a ten-fold dilution of sample may contain one tenth the total number of particles, it may contain almost as many bacteria as the undiluted mixture. This will occur if a few of the particles in the sample have unusually high numbers of cells adhering to them.

If the method is based on dilution of sample to an end point (e.g. the MPN and plate count assays), an additional error occurs at the end point itself. Thus, in a typical MPN assay, there is no differentiation as to whether the highest dilution with demonstrated growth contained originally one cell or one particle with many cells per tube. Similarly, in a plate count, a colony could be formed by a single cell or a group of cells bound together.

Particles may also interfere with the measurement technique itself. For example, in direct microscope counting, particles will obscure the cells, while with turbidity measurements, particles create a false positive signal. Particles may also contain substances which interfere with other types of measurements. An example of this problem is the interference of sulphur particles with the Folin and Ciocalteau reagent for protein measurement (Proteau and Silver, 1977). Finally, as mentioned in Section 5.8, the particles themselves may inhibit the growth of T. ferrooxidans.

From the above discussion, it appears significant to consider in greater detail the current knowledge of the association of T.ferrooxidans cells with particles.

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One type of association may involve entrapment. When T. ferrooxidans oxidizes ferrous ions in the presence of sulphate (both required for growth), precipitates which consist of crystalline jarosites and/or amorphous ferric hydroxysulphates are produced (Tuovinen and Carlson, 1979; Lazaroff et al., 1982). When these precipitates form, it is likely that some of the bacterial cells will become trapped within the precipitate.

There is also evidence for a direct interaction of T. ferrooxidans cells with mineral surfaces. Electron microscope studies on pyrite crystals placed in T. ferrooxidans cultures identified etching pits with the shapes of bacterial cells and of chains and clusters of bacterial cells (Bennett and Tributsch, 1978). Another electron microscope study (Berry and Murr, 1978) demonstrated the attachment of T. ferrooxidans cells to ore surfaces and indicated that the attachment occurred specifically at sulphide regions of the ore. This specific attachment also occurred with a Sulfolobus sp. and with T. thiooxidans. In an experiment, which studied the adhesion of T. thiooxidans to sulphur particles, Takakuwa et al. (1979) suggested that the adhesion process involved thiol groups since treatment of the cells with sulphydryl(SH)-binding agents (e.g. p-chloromercuribenzoate or iodoacetamide) prevented adhesion and the blocking could be reversed by the addition of an SH donor such as cysteine. The adhesion process was also apparently energy dependent since it was inhibited by the treatment of cells with metabolic inhibitors (e.g. 2,4-dinitrophenol, 2-heptyl-4-hydroxyquinoline-N-oxide, or sodium azide) or by depriving the cells of oxygen.

Studies involving surfactants have also produced evidence of the cell-surface interaction with T. ferrooxidans. Duncan et al.(1964) found that the presence of surfactants increased leaching rates. Production of a surfactant by T. ferrooxidans has also been reported (Grudev et al., 1978). However, no evidence has been obtained which demonstrates the induction of surfactant synthesis upon exposure of cells to a solid substrate (Tuovinen et al., 1978).

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Nevertheless, the extent of the interaction between particles and T. ferrooxidans cells appears to be quite significant. McGoran et al. (1969) estimated that in cultures of T. ferrooxidans in 9K medium, over 97% of the cells were associated with the particulate fraction of the medium, while Grudev et al. (1978) reported that over 80% of the cells in T. ferrooxidans culture had become attached to the mineral particles by the end of logarithmic growth. Adherence to particles can occur quite rapidly. Tuovinen et al. (1983) found that in T. ferrooxidans cell suspensions mixed with particles of pyrite, fluor-apatite, or glass beads, more than 25% of the cells were absorbed within five minutes, while Myerson & Kline (1983) observed that more than 50% of the cells were adsorbed within five minutes on to coal particles or glass beads.

Tuovinen et al. (1983) also noted that the degree of adsorption was independent of pH in the range of pH 1.0 to 4.0, except for glass which had a lower capacity as the pH decreased. Cells killed by ultraviolet light had similar initial adsorption rates, but total adsorption was reduced. Extraction of lipopolysaccharide from the cells had a more pronounced effect on adsorption. Because the experiments were performed with cells which had been washed several times with diluted sulphuric acid, it is unlikely that extracellular organic matter had a significant effect on the adsorption phenomena observed in these studies.

It also appears that the cells are adsorbed quite tenaciously. Myerson and Kline (1983) observed no desorption of T. ferrooxidans cells from coal or glass particles after 48 h. Increasing the pH (as high as pH 11) also had no effect on desorption. Moreover, Tuovinen et al. (1983) found that repeated washing of T. ferrooxidans cells bound to flowers of sulphur, fluorapatite, glass beads, or pyrite failed to remove the cells. McGoran et al. (1969) noted that the use of mild sonication and surfactants was ineffective in separating the cells from particulates, while LeRoux et al. (1973) reported that shaking (with or

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without Lubrol W or sodium pyrophosphate, agents found to release bacteria from flocs) reduced rather than increased the estimates of viable cells in populations of T. ferrooxidans.

The literature data confirm that association between T. ferrooxidans cells and particles is significant and occurs under most circumstances. Consequently, this association must be considered in any attempt to improve methodology for the enumeration of T. ferrooxidans.

7. CURRENT METHODS FOR DIRECT ENUMERATION OF T. FERROOXIDANS

7.1. Most Probable Number (MPN) Methods

Most MPN methods for T. ferrooxidans use the 9K medium of Silverman and Lundgren (1959). The literature appears to be devoid of reports concerning improvements over this medium for MPN testing. Freshly prepared medium has a pH of 3.0 to 3.6, contains 9000 ppm ferrous iron as an energy source, ammonium as a nitrogen source, phosphate as a phosphorus source, and also sulphate, potassium, calcium, and magnesium. The carbon source (CO_2) is supplied by the aerobic conditions of incubation and, therefore, shaking decreases the length of incubation requirements.

There are several difficulties with the MPN procedure for T. ferrooxidans and these are discussed in the following sections.

7.1.1 Inaccurate Counts Caused by the Adherence of Bacterial Cells to Particles in the Sample.

This problem, which is especially severe in slurries, has been discussed previously in Section 6. It is conceivable that errors of several orders of magnitude could occur because of this factor. To-date, little progress has been made towards a solution.

7.1.2. Difficulties in Interpreting a Positive Result

A positive result for growth in the liquid culture medium for T. ferrooxidans is usually assigned when the medium turns the colour characteristic of the bacterial oxidation of ferrous ion to the ferric form. However, with long incubation periods, this endpoint can be somewhat obscured due to oxidation of the ferrous ion by atmospheric oxygen.

In normal culture vessels, an increase in turbidity cannot be used to measure the growth of T. ferrooxidans because the medium itself contains a considerable amount of particles. However, Yunker et al. (1981) were able to measure the growth turbidimetrically by growing the organism in an electrochemical cell which continuously removed ferric precipitates. Such a technique would also prevent the inhibitory effect of ferric ions (Kelly and Jones, 1978). To make such an approach practical as an MPN technique,it would be desirable to have multiple cells constructed. They might appear similar to the cells used in a Bactometer (Bactomatic Inc.), a device which estimates cell numbers by measuring the change in electrical impendance as cells grow in a nutrient medium.

7.1.3. Requirement for Long Incubation Periods

T. ferrooxidans is a relatively slow-growing organism. Generation times ranging from 5.3 to 15 h (Beck, 1969; Silverman and Lundgren, 1959) have been reported for strains cultivated in the laboratory. However, when tests are conducted on natural isolates rather than laboratory cultures, the former strains will probably not be acclimatized to the environment of the MPN test. It is likely that longer generation times will occur and there may also be a significant lag period before the organism begins to grow. Thus, an incubation period of almost one week is required before the first indications of an MPN count are provided, while a minimum of three weeks is required to assign a negative (or absent) result to an MPN test.

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Clearly, this long incubation period is impractical for use in the process control of leaching operations and it creates difficulties in monitoring the risk of the environmental problems associated with the organism.

7.1.4. Toxicity Problems

When slurries are tested using the MPN method, the lower dilutions of a sample may introduce a significant amount of particulate matter into the test medium. The particles may contain toxic metals which can be leached out as the bacteria begin to grow. Inhibition of bacterial growth by these metals will result in a false low MPN count.

7.1.5. Specificity Problems

Most bacterial species will fail to produce a positive result in media such as 9K because of the low pH, the absence of organic nutrients, and because of their inability to oxidize ferrous iron. However, one exception is the bacterium Leptospirillum ferrooxidans (Norris and Kelly, 1978). This organism does differ from T. ferrooxidans in both its morphology and its inability to oxidize sulphur.

It is noteworthy that other bacteria may also grow in the presence of T. ferrooxidans in 9K medium. For example, there appears to be a close association between heterotrophic, acidophilic bacteria and T. ferrooxidans. This association has been observed even in cultures of T. ferrooxidans which had been thought to be pure (Silver, 1978; Harrison et al., 1980; Guay and Silver, 1975). Thus, Thiobacillus acidophilus was isolated from a culture of T. ferrooxidans by Guay and Silver (1975). It was later demonstrated (Kingma and Silver, 1981) that T. acidophilus can grow on pyruvate, which is excreted by T. ferrooxidans as an inhibitory end product. Arkesteyn and De Bont (1980) subsequently found that T. acidophilus, which did not grow on ferrous iron medium, could grow to a limited extent in the spent medium of T. ferrooxidans. They also observed that in mixed cultures of the two organisms, the numbers of each organism were almost equal.

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Recently, Johnson and Kelso (1983) have found that the contamination problem of acidophilic heterotrophs in T. ferrooxidans cultures may be eliminated by performing a subculture in the presence of high concentrations of copper. The T. ferrooxidans cells are resistant to those conditions whereas the contaminating bacteria are not.

7.2. Plate Count Methods

Plate count techniques for T. ferrooxidans suffer from many of the same problems as MPN testing. They are prone to the same errors caused by adsorption of cells to particulate matter (see Section 6.) and incubation periods are at least as long. On the other hand they are more precise because they do not have the high statistical sampling error which is a characteristic of all MPN tests, and they produce single colonies which can be used to obtain pure cultures of T. ferrooxidans. This is achieved with some difficulty because the cells adhering to inorganic precipitates do not disperse easily (Manning, 1975). However, colony appearance can assist in identifying the organism since only colonies of iron-oxidizing bacteria become first orange and then brown in colour (Manning, 1975).

One of the greatest difficulties in performing a plate count for T. ferrooxidans (in addition to slow growth and problems caused by particulate matter) originates with the solidifying agent. Agar (a galactose polysaccharide), the most commonly used solidifying agent in microbiological media, has been found to inhibit the growth of T. ferrooxidans (Manning, 1975; Tuovinen and Kelly, 1973; Beck, 1967; Bryner and Jameson, 1958).

In an effort to overcome the inhibitory effects of agar, Tuovinen and Kelly (1973) prevented the contact of T. ferrooxidans cells with the agar medium by initially collecting the cells on a membrane filter and then placing the filter on the agar medium. They found that by selecting the optimal pH, agar concentration, and brand of agar, an effective plate count procedure could be obtained. They also

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demonstrated that galactose (probably present in unpurified agar and produced by acid hydrolysis of the polysaccharide) was inhibitory to T. ferrooxidans.

Since this work, a number of improvements in the plate count method for T. ferrooxidans have occurred. Manning (1975) developed a medium using purified agar which did not require the use of membrane filters. It gave more rapid growth and larger colonies with distinctive morphological characteristics. However, he did not demonstrate that this medium gave high recoveries of the viable cells.

Mishra & Roy (1979) found that either carrageenan (Type 1) or agarose could be substituted for purified agar in Manning's medium. Carrageenan had the advantage that it could be autoclaved with the basal medium (agar had to autoclaved separately or else it would hydrolyze), while agarose was superior in producing large colonies. They also found that the presence of phosphate (0.025% w/v K_2HPO_4) inhibited growth as did agar medium which was prepared with a pH less than 3.0.

Subsequently, Mishra et al. (1983) modified the medium of Manning and using Bapco agar (Japanese), agarose, or carrageenan, they examined the plating efficiency of seven strains of T. ferrooxidans. Their formulation appeared to have excellent storage properties and they also demonstrated good recovery of viable cells. They suggested that any strain of T. ferroxidans could grow on this medium but some adaptation might be required.

A number of laboratories have used silica gel as a solidifying agent, but the preparation of good silica gel plates is tedious and tends to be unpredictable (Manning, 1975). An improved plate count procedure has been reported using membrane filters placed over silica gel plates prepared at pH 1.5 (Yunker et al. 1981)

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7.3. Direct Microscopic Counts

The use of direct microscopic counts for estimating T. ferrooxidans numbers has been limited because it is very tedious to distinguish the cells from particles of a similar size. Particles, which are generally present in high of quantities in most samples tested for T. ferrooxidans, also obscure the cells. Furthermore, many other species of bacteria are indistinguishable from T. ferrooxidans when viewed under the microscope.

These problems have been partially overcome with the development of a fluorescent antibody (FA) stain specific for T. ferrooxidans. Cells of T. ferrooxidans treated with this stain are clearly distinguishable when viewed under a microscope equipped with a fluorescent illuminator (Apel et al., 1976). Later Gates and Pham (1979) improved this procedure by developing an indirect fluorescent antibody stain which was simpler to prepare.

Although the fluorescent-antibody (FA) technique permits the visualization of T. ferrooxidans cells, it does not distinguish between dead and living cells. This shortcoming has been solved by Baker and Mills (1982) who combined the FA technique with the INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5 phenyl tetrazolium chloride) technique (i.e FAINT staining) for T. ferrooxidans. With the INT technique, actively metabolizing cells reduce INT to produce a formazan which accumulates as a dark red deposit inside the cell. Thus, after FAINT staining, actively respiring T. ferrooxidans cells would be viewed as fluorescent rods with intracellular formazan deposits. Results can be obtained after an overnight incubation (and potentially within several hours). A comparison of the FAINT method and the MPN test was made and indicated that the MPN test underestimated the number of viable T. ferrooxidans cells by one to three orders of magnitude.

While the FAINT technique appears to be very promising and overcomes the problems of the long incubation periods required for MPN

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and plate count tests, it does have disadvantages. The antibody preparation specific for T. ferrooxidans is not commercially available and requires animal facilities for production. Compared to typical MPN and plate count techniques, the FAINT technique is labour intensive and requires more skilled personnel.

Slurries also introduce problems. In the presence of large amounts of particles, many of the T.ferrooxidans cells will be obscured from view. Moreover, if the sample contains reducing agents such as ferrous ion, a non-biological reduction of the INT will occur. Some of the particles may also absorb the FA label non-specifically or cause auto-fluorescence problems (in which the particles themselves fluoresce when viewed in the microscope). Reduction of these problems requires sample pre-treatment such as adding bovine serum albumin (BSA); adding rhodamine isothiocyanate-conjugated BSA; or alternatively by washing the sample with a buffer solution. It has been observed, however, that these pre-treatments are not completely satisfactory. Finally, it was also found that biological reduction of INT was significantly slower in acid pH (Baker and Mills, 1982; Gates and Pham, 1979).

In conclusion, there is still a need to improve methodology for direct microscope counting of T. ferrooxidans

8. INDIRECT METHODS FOR ENUMERATION OF T. FERROOXIDANS

The difficulties, described in previous sections, have prompted investigators to estimate populations by correlating T. ferrooxidans cell numbers with various biomass or metabolic indicators. Examples of this approach include measurement of (a) protein content (Proteau and Silver, 1977; Kingma and Silver, 1979), (b) total organic nitrogen (Gormley and Duncan, 1974), (c) radioactive CO_2 fixation (Brierley, 1977), (d) adenosine triphosphate (ATP) content (Tuovinen and Sormunen, 1979), (e) oxygen uptake rate (Brierley, 1978), and (f) iron oxidation rate (Gol'braikht, 1978).

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A major problem with the tests which are based on measuring components of biomass, is that they do not distinguish between dead and living cells. These components can also be associated with detrital material. On the other hand, a major concern with tests based on the measurement of metabolic activities is that they cannot distinguish whether a response is generated by a few very active cells or many weakly active cells.

A noteworthy exception to these problems is the ATP (adenosine triphosphate) assay. ATP is found only in living cells (most of a cell's ATP is lost quickly after death) and is not an integral part of non-living systems. Furthermore, it has been found that the amount of ATP in a cell is directly proportional to its mass (Holm-Hansen, 1973). These characteristics, together with the fact that the test is rapid, is easy to perform, and is relatively sensitive (e.g., it is possible to measure as few as 10^3 bacteria in routine testing), make the ATP assay attractive for application to T. ferrooxidans studies. The technique would probably be used more frequently, but difficulties have been encountered with ATP extraction and measurement in the presence of high levels of iron compounds and heavy metals in the samples (Tuovinen and Sormunen, 1979). Furthermore, the methods, which were described to reduce these problems, were cumbersome and probably somewhat inaccurate, especially when they are compared to more recent improvements in methods for ATP extraction (Afghan et al., 1978).

None of the indirect techniques for estimating the numbers of T. ferrooxidans in a sample, including the ATP assay, measure T.ferrooxidans specifically. Therefore, if T. ferrooxidans is not the major component of the biomass in the sample, false positive results will occur. An approach to overcome this specificity problem is to combine an indirect technique with an enumeration procedure which can provide the required specificity.

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An example of this strategy is the FAINT technique, described in Section 7.3., in which the INT assay, an indirect technique to measure microbial dehydrogenase activity, is combined with the fluorescent antibody (FA) technique which is specific for detecting T. ferrooxidans.

Another approach, described by Silverman and Munoz (1979), although not applied to T. ferrooxidans enumeration, is the combination of electrical impedance measurements (a rapid, automated, but not specific, methodology for measuring microbial growth rates) with an MPN technique specific for fecal coliforms.

The above examples demonstrate the potential value of applying indirect methodology in order to improve direct enumeration techniques. It is most probable that this general principle could be further applied to methodology for the enumeration of T. ferrooxidans.

9. CONCLUSIONS

We have undertaken a critical assessment of the current state-of-the-art of enumeration methods for T. ferrooxidans in slurries of particles. This has included a brief review of the various factors in actual samples of slurries or process mixtures, which may affect the growth of the organism, and, therefore, may influence the accurate measurement of the number of viable T. ferrooxidans cells in the samples.

We have identified the major limitations of existing techniques. A key factor, inherent in all direct enumeration methods, is the interference caused by the close association existing between T. ferrooxidans cells and insoluble substrates (sulphides) or other particulate matter. The most probable number (MPN) methods are also limited by difficulties in the interpretation of the MPN endpoint, the long incubation time required, and the possible lack of specificity of the test. Toxic components in samples are also of concern.

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Similar weaknesses are identified with plate count techniques. Although there have been improvements with the specificity of the test, other problems arise from the inhibition of T. ferrooxidans growth by factors in the solidifying agent e.g. agar. Solutions to this problem have been reported, but their value for the enumeration of unadapted strains of T. ferrooxidans from natural samples has yet to be assessed.

Methods based on microscopic, direct count procedures e.g. the FAINT technique are still sensitive to the presence of particles. They generally require specialized reagents and highly skilled personnel. Their application as routine counting procedures is questionable.

The use of indirect methods such as those based on the measurement of specific metabolites has been reviewed. Their benefit to the current project may be as end-point indicators or to monitor the effect of various test protocols on the viability of T. ferrooxidans cells.

The key factor which must be addressed at the outset of an experimental program will be the identification of a method for the release of T. ferrooxidans cells from their association with particles. A successful and simple protocol must overcome this initial hurdle.

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APPENDIX A

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APPENDIX B
(Supplement to Bibliography)

leachates are observed, the microbial activity within the tailings is well established. It is then more difficult to alleviate the problem. Early-warning techniques are required to permit management personnel to use control measures on tailings, in advance of the observed chemical changes. This should not only be more effective but also economically more attractive to the mining companies.

Related to both the leaching processes and acid mine drainage is the need to develop a further understanding of the microbial ecology of T. ferrooxidans. Evidence has been obtained which suggests a synergistic relationship between T. ferrooxidans and acidophilic heterotrophs (Kingma and Silver, 1981; Arkesteyn and DeBont, 1980). However, there is little knowledge concerning microorganisms which are antagonistic to the growth of T. ferrooxidans. Furthermore, little has been described about the biological mechanisms involved in the successive changes in microbiological populations which eventually lead to a predominance of acidophilic organisms.

Progress in acquiring this key information has been greatly impeded by the inadequate methodology for enumeration of T. ferrooxidans cells in slurries (Brierley, 1978). Nevertheless, many research groups have used procedures such as the most probable number (MPN) technique in order to estimate the magnitude of populations of T.ferrooxidans. In some instances, reservations about the methodology have been noted by the authors (Schubert and Miller, 1982) and a recent study has indicated that the MPN procedure was unreliable when used to enumerate T.ferrooxidans in pyrite-containing tailings (Silver and Sanmugasunderam, 1983). In later sections of this report, we identify why current methods yield inaccurate results and require very long incubation periods. Consequently, until an improved method is available to enumerate T.ferrooxidans in samples containing particles, studies to assess the effects of genetic, physiological and ecological forces on the behaviour of T.ferrooxidans will be awkward to perform and difficult to interpret.

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